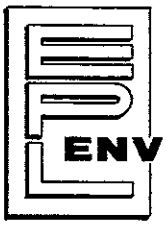

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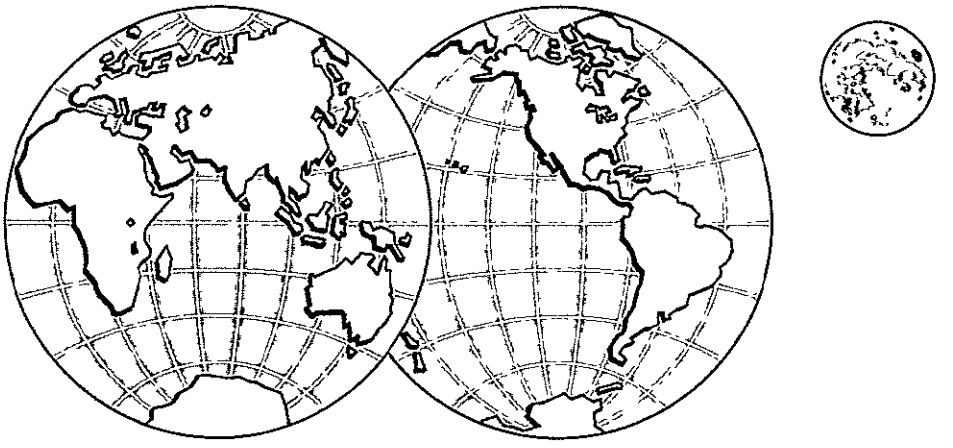

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UNIVERSITY OF CALIFORNIA, BERKELEY

METHODOLOGY FOR ESTIMATION OF TOTAL BODY COMPOSITION IN LABORATORY MAMMALS

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INTRODUCTION

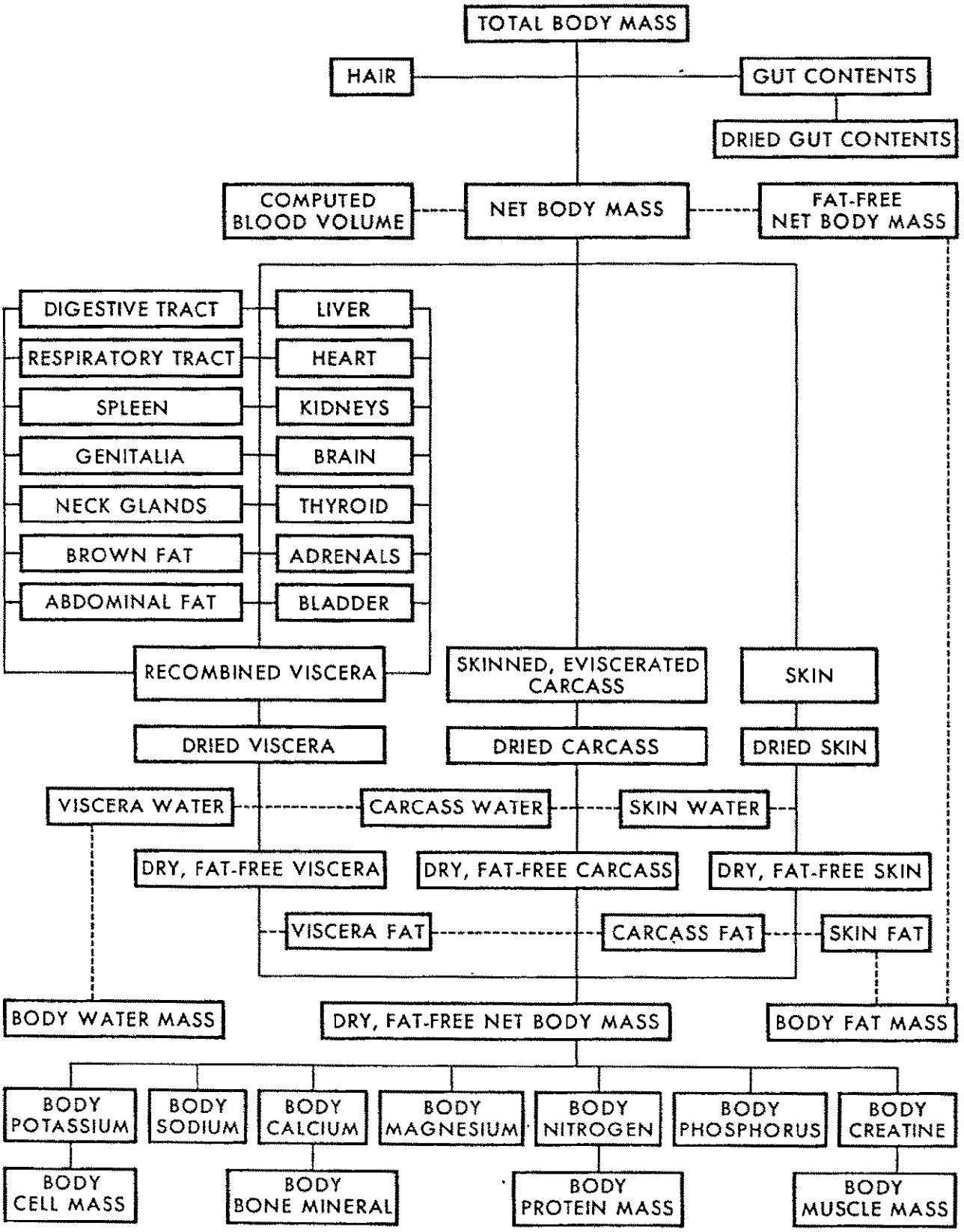
Galileo, more than 300 years ago, postulated that the morphology of organisms inhabiting the surface of the Earth is influenced by the loading imposed on them by the force of gravity (3). The concept of structural scale effects as a function of organism size has been elaborated upon over the years, and is now accepted as a fundamental biological principle (4, 13). However, many of the quantitative relationships that have been derived are based on literature data for various species examined by a wide array of investigators using different techniques, with a consequent large degree of variance in the derived relationships.

As part of a study to examine the scaling of metabolic rate as a function of body size and body composition in small terrestrial mammals, we have developed a standardized dissection and chemical analysis procedure for individual animals of several species in the size range mouse to monkey (15 g to 15 kg). The standardized procedure permits rigorous comparisons to be made both interspecifically and intraspecifically of organ weights and gross chemical composition in mammalian species series, and has been applied successfully to laboratory mice, hamsters, rats, guinea pigs, and rabbits (10), as well as to macaque monkeys. A schema of the procedure is shown in Fig. 1, and detailed descriptions are given in the following sections.

The masses of the principal organs of the animal are determined individually by weighing as they are dissected free. However, in order to determine water and fat content, the organs are collected quantitatively on three aluminum-foil trays for subsequent drying and fat extraction.

The containment integument of the animal, the skin, is placed on one tray. All of the internal organs, or viscera, are collected on a second

Fig. 1. EPL Schema for Animal Dissection and Biochemical Analysis



tray. The remaining skinned, eviscerated carcass, which is largely the musculoskeletal system and which may be regarded as the weight-bearing organ of the body, occupies the third tray, and for convenience is termed the carcass.

The water and fat content of each of the three categories, skin, viscera and carcass are determined separately, and the sums provide the total body water and fat masses. The scaling of these three categories as a function of body size is of interest in itself.

The dried, defatted tissues are combined, comminuted, and thoroughly mixed to produce a uniform, dried, defatted total body powder. Powder samples are then digested for chemical analysis. Among the constituents determined are body potassium, sodium, calcium, magnesium, nitrogen, phosphorus, and creatine. From these data, values may be computed for such entities as body cell mass, bone mineral mass and protein mass.

DISSECTION AND ORGAN MASS DETERMINATIONS

Animal sacrifice is made by etherization within a closed container, and the time of day is recorded. Immediately following cessation of heart beat the total body mass is determined to 0.01 gram. Any urinary or fecal fouling of the hair is wiped off, and the hair is removed with electrically powered clippers and discarded. The depilated body mass is determined to 0.01 gram.

The animal is placed in the prone position on a pre-weighed, laboratory-fabricated, aluminum-foil tray, which ultimately becomes the carcass tray. Dissection and organ mass determinations of the depilated body proceed in the order shown below. Each component is weighed to 0.001 gram, and any anatomical anomalies are noted. For masses of less than 150 grams, a Mettler Model P160N balance is used. For masses up to 500 grams, a Torsion Model DLT5 balance is used.

1. Skin. A mid-line dorsal incision through the skin is made by surgical scissors and scalpel from the posterior tip of tail to the anterior tip of the nose. The skin is separated manually from the subcutaneous tissue. Further separation is carefully accomplished with the aid of a scalpel and blade or surgical scissors. Sharp dissection is particularly helpful in portions of the ventral body and the anterior and posterior extremities. In this manner, the skin may be removed intact. The external ears (auricles) are included with the skin. Particular care is needed in the perianal region (genitalia and anus) in order to prevent disturbance of the underlying structures. The skin is weighed to 0.001 gram, and is then cut into pieces not exceeding 2x2 cm in size, which are spread on a separate, pre-weighed aluminum-foil tray, the skin tray, for subsequent freeze-drying and fat extraction.

2. Brown Fat. With the animal still in place in the prone position, the discrete bilobed interscapular fat body is removed by sharp dissection using surgical scissors and forceps, and is weighed to the nearest 0.001 gram. The brown fat is placed on a second, pre-weighed aluminum-foil tray, the viscera tray.

3. Tail. The tail is removed at the level of the insertion of the caudal vertebrae in the pelvic area, is weighed to 0.001 gram; and is eventually added to the carcass tray. The carcass is turned to a supine position. Cutting and spreading the shoulder musculature ventrally aids in positioning the body for further dissection.

Using surgical scissors aided by a tissue forceps, the abdomen is opened by a mid-line incision between the external genitalia and the xiphoid process of the sternum. Transverse incisions of the abdominal musculature are also made to expose the underlying organs. The sternum from xiphoid process to manubrium, with attached musculature, is removed with scissors to

expose the thoracic cavity, and is eventually added to the carcass tray.

4. Heart. Cutting the pericardium exposes the heart, which is separated at its junction with the major blood vessels. All chambers of the heart are opened, and the blood is drained into the thoracic cavity. The heart is weighed to 0.001 gram, and then is added to the viscera tray. Up to this point no major hemorrhaging may be anticipated. The blood which enters the thoracic cavity is removed by syringe and saved to add to the viscera tray.

5. Neck glands. All discrete glandular tissue in the superficial region of the neck, consisting mainly of salivary glands and lymph nodes, is removed, together with the thymus from the upper chest. These tissues are combined as the neck glands, weighed to 0.001 gram, and added to the viscera tray. The portion of the salivary and lacrimal glands imbedded within the musculature of the mandible and maxilla is not included and remains with the carcass.

6. Thyroid. The neck musculature is separated by blunt dissection to expose the thyroid gland and trachea. The thyroid is dissected free using irridectomy scissors, is weighed to 0.0001 grams, and is added to the viscera tray.

7. Respiratory Tract. The mandible is split medially with scissors and is further broken manually, exposing the cranial portion of the respiratory tract. The ventral rib cage is removed, to facilitate extirpation of the lungs, and is eventually weighed with the eviscerated carcass. Blunt dissection is used to separate the trachea from the esophagus. The larynx is excised intact with the trachea and is manually pulled away from the remaining tissue surrounding the esophagus. The respiratory tract, comprising the lungs, trachea and larynx, is removed, weighed to 0.001 gram, and added to the viscera tray. In most cases the removal of the entire respiratory tract can be accomplished in this manner with minimal scissor separation of blood

vessels. The presence of any pleural adhesions should be noted.

The tongue, pharynx and esophagus are separated from the body cavity and left intact to the level of entrance into the diaphragm. The diaphragm is incised to free the esophagogastric junction, and the esophagus is laid over to the left side.

8. Liver. The liver is separated with scissors from the adhering ligaments, blood vessels and pancreatic tissue. The liver is removed, weighed to 0.001 gram, and added to the viscera tray. With the liver removed, the digestive tract can be rotated to the right side and the spleen is exposed.

9. Spleen. The spleen is readily apparent, and can be dissected free easily. The spleen is removed, weighed to 0.001 gram, and added to the viscera tray.

10. Adrenals and Kidneys. The left adrenal and kidney are removed separately in that order. The digestive tract is then moved toward the left to expose the right adrenal and kidney, which are likewide removed separately. The kidneys are excised at the hilus and removed from the surrounding abdominal fat. The adrenals and the kidneys are weighed to 0.001 gram as separate entities, and then added to the viscera tray.

11. Bladder. The urinary bladder is picked up with tissue forceps and separated from adhering tissue. A curved hemostat is fixed to the trigone and the bladder is excised with surgical scissors. The bladder is removed and cut open over a small beaker to collect any urine, which is then weighed to 0.001 gram, and discarded. The empty bladder is weighed to 0.001 gram, and is added to the viscera tray.

12. Genitalia. To assist in removing these organs, the symphysis pubis is divided by scissors and broken open manually. The lower digestive tract, starting at the anus, is dissected free to expose the internal genitalia.

For male animals, all primary and secondary sex organs, comprising the testes, epididymis, vas deferens, vesicular glands, prostate, coagulating glands and penis, are dissected free and weighed to 0.001 gram as a single entity. For the female animals, all primary and secondary sex organs, comprising the ovaries, oviducts, uterus, cervix, vagina, vulva and associated secretory organs, are dissected free, weighed to 0.001 gram. After weighing, the genitalia are added to the viscera tray. Perigonadal fat is removed and incorporated with the abdominal fat.

13. Digestive Tract, Tongue to Rectum. The anus and rectum are separated from the underlying abdominal fat and stripped cranially. The total digestive tract including the tongue is easily separated from adhering blood vessels and membranous tissue. The adipose tissue closely applied to the tract is stripped away as cleanly as possible, and is incorporated with the abdominal fat. The pancreas is diffuse and is included as part of the digestive tract. The intact digestive tract is weighed to 0.001 gram and cut into 10 cm sections. The gut contents of each section are squeezed out manually and collected on a pre-weighed plastic weighing tray for subsequent dry mass determination. The empty digestive tract sections are collected, weighed to 0.001 gram, and added to the viscera tray.

14. Gut Contents. The plastic weighing tray containing the gut contents is dried for 24 hr in a 110°C oven, weighed to 0.001 gram, and discarded.

15. Abdominal Fat. Perirenal, perigonadal and mesenteric fat deposits are removed by sharp dissection. The combined abdominal fat deposits are weighed to 0.001 gram, and are added to the viscera tray.

16. Brain. Soft tissue is removed from the calvarium, and bone incision is made with scissors or Stryker saw. The complete brain is removed by blunt dissection and severed at the junction of the medulla with the spinal cord. The brain is weighed to 0.001 gram, and added to the viscera tray.

17. Skinned, Eviscerated Carcass. The remaining carcass is weighed to 0.01 gram, and is cut into pieces not exceeding 2x2x2 cm in size which are kept in the carcass tray. The tail is also cut into 2-cm segments and added to the carcass tray.

Thus, the total dissected material is distributed to three aluminum-foil trays: one containing the depilated skin, one containing the recombined viscera of the animal, and the third containing the skinned, eviscerated carcass plus tail. The aluminum-foil trays are stored at -20°C for subsequent analysis.

It bears emphasis that the dissection procedure should be carried out carefully, but as rapidly as possible in order to minimize evaporative losses. In our laboratory, experience has shown that an animal of the smaller species can be processed within an hour, while an animal of the larger species requires up to two hours for complete dissection.

The result of the total dissection procedure is three aluminum-foil trays containing the following tissue systems:

Tray 1. Depilated skin.

Tray 2. Recombined viscera, comprising brown fat, heart, neck glands, thyroid, respiratory tract, liver, spleen, adrenals, kidneys, bladder, genitalia, digestive tract, abdominal fat, brain, and blood.

Tray 3. Skinned, eviscerated carcass plus tail.

The three trays are stored at -20°C for subsequent biochemical analysis. For convenience Tray 1 is termed Skin, Tray 2 is termed Viscera, and Tray 3 is termed Carcass.

The animal dissection data are recorded on a form shown in Fig. 2, which includes actual values from the dissection of a male rat of 82 days

Fig. 2. Animal dissection data form, and example values.

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ANIMAL DISSECTION DATA

Animal Number 5 Time 1115 - 1210 Date 29 June 1979
(Day, Month, Year)

Animal Species Rat Sex Male Age 82 d

	Total	Tare	Net
1. Total Body Mass (g)			372.43
2. Depilated Body Mass (g)			365.93
3. Brown Fat (g)			--
4. Skin (g)	77.02	9.82	67.20
5. Heart (g)			1.142
6. Blood Sample (g)			--
7. Neck Glands (g)			2.853
8. Thyroid (g)			0.023
9. Lungs and Trachea (g)			2.573
10. Liver (g)			18.919
11. Spleen (g)			0.691
12. Adrenals (g)			0.096
13. Kidneys (g)			3.806
14. Bladder Contents (g)			0.132
15. Bladder, empty (g)			0.083
16. Genitalia (g)			13.970
17. Abdominal Fat (g)			8.655
18. GI Tract, Tongue to Rectum (g)			25.744
19. GI Tract, empty (g)			13.245
20. Tail (g)			12.981
21. Brain (g)			2.001
22. Eviscerated Carcass (g)	216.38	23.75	192.63
23. Carcass plus Tail (g)			205.61
24. Viscera	85.89	12.07	73.82
25. Recombined Mass, with blood but without hair and gut and bladder contents (g)			346.63
26. GI Contents, wet (g)	22.09	10.07	12.02
27. GI Contents, dry (g)	12.81	10.07	2.74

Remarks:

of age as an example. Item 24 on the form, the Viscera, is the weighed mass of the recombined viscera plus blood, and is used only as a rough check on the recovery of all the dissected tissues. Similarly, the wet weight of the GI contents, Item 26, is used only as a rough check on the GI Contents Mass actually computed as the difference in masses of the GI Tract, full and empty. Finally, the Brown Fat Mass is not shown for the example animal because it was not measured separately in this instance.

COMPUTATION OF CORRECTED ORGAN MASSES

Fig. 3 is a sample of the form used to compute corrected values for the individual organ masses, incorporating an approximate correction for both blood content and tissue drying during dissection. This is done by calculating the blood volume of the animal as 7 per cent of the net body mass (Item 27), and subtracting the computed blood volume from the net body mass to obtain the drained net body mass (Item 29). Brody (1) showed that blood volume is an isogonic (first power) function of body mass over a wide range of species body sizes, and 7 per cent of the net body mass seems to represent a reasonable mean value.

The measured organ weights from Fig. 2 are all rounded to the nearest 0.01 g, and are entered in the first column of Fig. 3 (Items 4-15, 17, 21, 23-25). The drained net body mass (Item 29) is divided by the sum of the measured organ weights (Item 30) to arrive at a "Hydration Factor", Item 31. Each measured organ weight is then multiplied by the "Hydration Factor" to yield a Corrected Organ Mass (Column 2), which represents an approximation of the fresh, blood-free mass of each organ.

If no evaporative water loss or blood loss at all were to occur from each organ during the dissection procedure, the "Hydration Factor" would be defined by drained net body mass divided by the net body mass. The latter is equivalent to the sum of all of the individual organ masses together with their blood content. Inasmuch as the whole-body blood mass is arbitrarily taken as 7% of the net body mass, the "Hydration Factor" in this hypothetical instance would always be 0.93000. Multiplying each organ mass by the "Hydration Factor" would yield a reasonable approximation of the drained organ mass, based on the assumption that each organ contains 7% blood.

Fig. 3. Organ mass computations form, and example values.

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UNIVERSITY OF CALIFORNIA, BERKELEY

ORGAN MASS COMPUTATIONS

Animal Number 5 Date 29 June 1979
(Day Month Year)

Animal Species Rat Sex Male Age 82 d

		Mass (g)	Corrected Mass (g)	Total Body Mass (%)	Net Body Mass (%)
1.	Total Body Mass	372.43	—	100.00	—
2.	Depilated Body Mass	365.93	—	—	—
3.	Hair (1. minus 2.)	6.50	—	1.75	—
4.	Brown Fat	—	—	—	—
5.	Heart	1.14	1.10	0.30	0.31
6.	Neck Glands	2.85	2.75	0.74	0.78
7.	Thyroid	0.023	0.02	0.01	0.01
8.	Lungs and Trachea	2.57	2.48	0.67	0.70
9.	Liver	18.92	18.24	4.90	5.16
10.	Spleen	0.69	0.67	0.18	0.19
11.	Adrenals	0.096	0.09	0.02	0.03
12.	Kidneys	3.81	3.67	0.99	1.04
13.	Bladder, empty	0.083	0.08	0.02	0.02
14.	Genitalia	13.97	13.47	3.62	3.81
15.	Abdominal Fat	8.66	8.33	2.21	2.35
16.	GI Tract, full	25.74	—	—	—
17.	GI Tract, empty	13.25	12.77	3.43	3.61
18.	GI Contents	12.49	—	—	—
19.	Bladder Contents	0.13	—	—	—
20.	Gut Contents	12.62	—	3.39	—
21.	Brain	2.00	1.93	0.52	0.55
22.	Corrected Viscera (Sum 4.-15.,17., 21.)	—	65.60	17.61	18.56
23.	Skin	67.20	64.78	17.39	18.34
24.	Tail	12.98	12.51	3.36	3.54
25.	Eviscerated Carcass	192.63	185.69	49.86	52.56
26.	Recombined Mass	346.63	—	—	—
27.	Net Body Mass (2. minus 20.)	353.31	—	94.86	100.00
28.	Blood (7% of 27.)	24.73	24.73	6.64	7.00
29.	Difference (27. minus 28.)	328.58	—	—	—
30.	Sum, 4.-15., 17., 21., 23.-25.	340.87	—	—	—
31.	Hydration Factor (29./30.)	0.96395	—	—	—
32.	Corrected Sum 22.-25., 28	—	353.31	94.86	100.00

On the other hand, if all of the water had evaporated from each organ during the dissection, and before they were weighed, the "Hydration Factor" would be defined by drained net body mass divided by the dry net body mass, which is equivalent to the sum of all of the individual dry organ masses. In the case of the example animal shown in Fig. 3, using the dry net body mass determined as described in the next section, the "Hydration Factor" would have been 328.58 g/123.87 g, or 2.6526.

Thus, the "Hydration Factor" can vary from a minimum value of 0.93000, if no evaporation or blood loss occurs during dissection, to a maximum value defined by the ratio of the drained net body mass to the dry net body mass, which is of the order of 3 and dependent on the relative fatness of the animal. In our experience, with our dissection procedure, the "Hydration Factor" is usually in the range of 0.96 to 0.98, implying a water loss of 2 to 3% during the dissection. We have also found that computing and using five significant figures for the "Hydration Factor" minimizes rounding errors in computing corrected organ masses.

The corrected masses for Items 4-15, 17 and 21 are summed to provide the Corrected Viscera Mass (Item 22). The corrected masses of the tail and skinned, eviscerated carcass are shown separately in Column 2 of Fig. 3 for record purposes. However, these two masses are summed to provide the Corrected Carcass Mass used in subsequent calculations.

Columns 3 and 4 of Fig. 3 show the corrected organ masses as percentages of total body mass and of net body mass, respectively. It may be added that we follow the practice of arbitrarily adjusting Item 15, "Abdominal Fat", by the very small numerical quantity necessary to obviate rounding error in all the totals involved.

DETERMINATION OF BODY WATER MASS, BODY FAT MASS, AND FAT-FREE NET BODY MASS

The trays containing the frozen pieces of tissue at -20°C are placed on separate shelves in the chamber of a vacuum freeze-dry apparatus (Virtis Company Model USM-15), together with a 50-ml beaker containing about 30 ml of distilled water and a temperature probe. The chamber door is closed and refrigerant is circulated through the shelves to produce a temperature of -50°C to -60°C, as measured by the beaker temperature probe. Approximately 3-4 hr are usually required to attain temperature equilibrium. When it is reached, vacuum is applied to the chamber and the refrigerant circulation is shifted from the shelves to the apparatus condenser. The vacuum and refrigeration are maintained for 2-3 days, depending on the mass of material being dried.

Completion of the drying process is judged from the disappearance of all the water from the 50-ml beaker. When the water is gone, the shelves are heated to 50°C and are left at this temperature overnight before the vacuum is released to remove the tissue trays. The trays are weighed to obtain the dried tissue mass for each of the three systems as the difference from the original empty tray weights. The sum of the three dried tissue masses yields the dry net body mass, as shown in Fig. 4.

The dried tissues are transferred quantitatively to Soxhlet thimbles and stored in a desiccator until the fat extraction procedure is ready to begin. The thimbles plus dry tissue are weighed, and the thimbles are inserted into the extraction chamber of a Soxhlet apparatus charged with petroleum ether (Mallinckrodt #6128 for fat extraction, boiling range 30-60°C). The tissues are extracted continuously for 3 days, after which time the

Fig. 4. Dry net body mass and body fat mass measurements form, and example values.

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DRY NET BODY MASS AND BODY FAT MASS MEASUREMENTS

Species Rat Animal No. 5 Sex Male Age 82 d

Dry Net Body Mass

Tissue	Drying Tray Empty (g)	Tray + Dry Tissue (g)	Dry Tissue Mass (g)
Skin	9.82	38.56	28.74
Viscera	12.07	40.84	28.77
Carcass	23.75	90.11	66.36
		Dry Net Body Mass (g)	123.87

Body Fat Mass

Tissue	Thimble + Dry Tissue (g)	Thimble + Defatted Tissue (g)	Fat Mass (g)
Skin	45.96	31.96	14.00
Viscera	45.42	28.71	16.71
Carcass	85.12	71.48	13.64
		Body Fat Mass (g)	44.35

Dry Fat-Free Net Body Mass

Tissue	Dry Tissue Mass minus Fat Mass (g)
Skin	14.74
Viscera	12.06
Carcass	52.72
Dry Fat-Free Net Body Mass (g)	79.52

thimbles are removed from the Soxhlet extraction chamber and placed in empty, open glass jars. The jars are placed in a boiling-water bath for 2-3 hr to drive off most of the petroleum ether from the tissues, and then the jars are placed in an oven set at 110°C overnight. The thimbles are removed and weighed, and the fat contents of the three tissue systems are computed as the difference between pre- and post-extraction thimble weights. The sum of the three fat contents yields the body fat mass, as shown in Fig. 4.

The dry, fat-free mass for each of the three tissue systems is obtained from the data in Fig. 4 as the difference between the dry tissue mass and fat mass. The sum of the three dry, fat-free tissue masses yield the dry, fat-free net body mass, as shown in Fig. 4.

Appropriate data from Figs. 3 and 4 are combined as shown in Table 1 to obtain the body water and body fat distribution in the subject animal. The water mass of each of the three tissue systems analyzed is computed as the difference between the corrected tissue wet masses from Fig. 3 and the dry tissue masses from Fig. 4. In addition, the calculated blood volume from Fig. 3 is assumed to be 100% water -- a simplifying assumption with a small, but acceptable, error. The sum of the four water masses is taken as the net body water mass of the animal.

The fat-free wet mass of each of the three tissue systems analyzed is calculated as the difference between the corrected tissue wet masses from Fig. 3 and the fat masses from Fig. 4. In this instance, the calculated blood volume is assumed to be fat-free, and is simply added to the three fat-free tissue system masses to yield the fat-free net body mass.

Table 1 also contains the fractional distribution of the body fat and water masses among the tissue systems considered. In addition, the fat and water contents are shown as percentage values of body mass.

Table 1. Computation of body water and fat distribution in example animal.

	Skin	Viscera	Carcass	Blood	Net Body
Wet Mass, g (Fig. 3)	64.78	65.60	198.20	24.73	353.31
Dry Mass, g (Fig. 4)	28.74	28.77	66.36	--	123.87
Water Mass, g	36.04	36.83	131.84	24.73	229.44
Dry, Defatted Mass, g (Fig.4)	14.74	12.06	52.72	--	79.52
Fat Mass, g (Fig. 4)	14.00	16.71	13.64	--	44.35
Fat-Free Wet Mass, g	50.78	48.89	184.56	24.73	308.96
Body Fat Fraction	0.316	0.377	0.307	--	1.000
Body Water Fraction	0.157	0.161	0.575	0.107	1.000

CHEMICAL ANALYSIS OF DRY, FAT-FREE NET BODY MASS

All the dried, defatted tissue pieces for each animal are collected quantitatively from the Soxhlet thimbles and comminuted in a Wiley mill (Arthur H. Thomas Model ED-5, 2 mm screen size). The pulverized tissue material is combined in a large, screw-topped glass jar, and is thoroughly mixed by hand tumbling of the jar. The combined material is passed through the Wiley mill a second time, followed by thorough mixing again. Experience has shown that 1-gram samples of the dry, defatted, net-body powder yield consistently reproducible results from subsequent chemical analyses.

Kjeldahl Digestion

The Kjeldahl digestion is based on the method of Sanui and Pace (12). Somewhat more than 1 gram of dry, defatted, net-body powder is placed in a desiccator overnight. Approximately 1 gram of the desiccated powder is weighed out accurately to 1 mg in a disposable plastic weighing tray (Scientific Products Catalog No. B2045-10) using a Mettler Model B6, semi-micro analytical balance. The powder is added to a 500 ml Pyrex Kjeldahl digestion flask (Corning No. 5420) previously calibrated to contain 550 ml by a ring marked on the neck. Next, 20 ml of concentrated H_2SO_4 (sp.gr. 1.84), 0.5 g of a 25:1 (weight for weight) mixture of lithium sulfate ($Li_2SO_4 \cdot H_2O$) and anhydrous copper sulfate ($CuSO_4$) catalyst powder, and 6-10 quartz boiling chips are added to the flask. The mixture is digested at boiling temperature until it clears, usually 4-6 hr.

The flask is allowed to cool for 30 min at room temperature. A volume of 0.5 ml reagent grade 30% hydrogen peroxide (H_2O_2) is added, and boiling is resumed for another 30-60 min. The flask is cooled, high-quality distilled

water is added to bring the volume up to the 550 ml calibration mark, and the contents of the flask are well mixed. A 25-ml portion of the unknown sample diluted Kjeldahl digest is stored in a small polyethylene screw-topped bottle for subsequent elemental analyses.

Duplicate blank digests are prepared in parallel with the unknown samples being analyzed. The blank digests contain all the ingredients, save for the whole-animal powder, and are treated in the same manner as described above.

Duplicate known, control samples are also digested in parallel with the unknown samples and blanks. The control sample is an aqueous solution made up in a 1 liter volumetric flask in the following manner. A quantity, accurately weighed to 1 mg, of 0.45 grams of reagent-grade magnesium metal shavings (Mg) and 30 grams of reagent-grade calcium carbonate ($CaCO_3$) are placed in a 1-liter volumetric flask with 90 ml of concentrated hydrochloric acid (HCl). The mixture is swirled until all the material is in solution. Approximately 500 ml of distilled water are added. A quantity, accurately weighed to 1 mg, of reagent-grade of each of the following compounds is added to the flask: 60 grams of urea ($(NH_2)_2CO$); 25 grams of concentrated (85%) phosphoric acid (H_3PO_4); 4.5 grams of potassium chloride (KCl); and 2.5 grams of sodium chloride (NaCl). The contents of the flask are swirled until all the material is in solution, and the volume of the flask is made up to 1.000 liter with distilled water. The control solution contains approximately 2.0 M nitrogen, 0.30 M calcium, 0.22 M phosphorus, 0.060 M potassium, 0.043 M sodium, and 0.019 M magnesium. An accurately measured volume of 5.0 ml of the control solution is digested, in duplicate, in parallel with the unknown samples and blanks as described above, to provide a control diluted Kjeldahl digest.

Nitrogen Determination

Nitrogen determination in the Kjeldahl digests is based on the ammonia procedure of Grunbaum and Pace (5). The method depends on a timed color reaction produced by the serial addition of two reagent solutions to the sample solution to be analyzed. Each of the two reagent solutions must be freshly prepared for each day's analyses.

Phenol color reagent is made by dissolving 5.0 grams of reagent-grade phenol (C_6H_5OH) and 25 mg of reagent-grade sodium nitroferricyanide ($Na_2Fe(CN)_5NO \cdot H_2O$) in distilled water, to make 500 ml in a volumetric flask. Alkaline hypochlorite reagent is made by dissolving 2.5 grams of sodium hydroxide (NaOH) and 1.0 ml of 5% sodium hypochlorite reagent (NaOCl; Matheson, Coleman and Bell No. SX0610) in distilled water, to make 500 ml in a volumetric flask. The alkaline hypochlorite reagent is sensitive to light and must be kept in a brown bottle and refrigerated when it is not being used.

A volume of 5.00 ml of phenol color reagent is pipetted into a 17x100 mm capped polypropylene test tube (Falcon No. 2059), and 0.020 ml of the unknown diluted Kjeldahl digest sample are added. Immediately, 5.00 ml of alkaline hypochlorite reagent are pipetted into the tube, which is capped, shaken, and placed in a water bath at 85°C for exactly 15 min. The tube is immediately cooled under running tap water for 5 min, inverting the tube several times while cooling. A 1-cm cuvette is filled with the resulting blue solution, and the optical density at 630 nm is read in a spectrophotometer (Bausch and Lomb Spectronic 70).

Duplicate 0.020 ml samples of the blank and the control solution diluted Kjeldahl digests described in the preceding section are analyzed colorimetrically in the same manner as above.

A standard curve for optical density as a function of nitrogen concentration is prepared by analyzing 4 solutions of ammonium sulfate ($(NH_4)_2SO_4$) of different, known concentrations. These are prepared by dissolving an accurately weighed quantity of 2.0 grams of $(NH_4)_2SO_4$ in 1% H_2SO_4 and making the final volume up to 1.000 liter in a volumetric flask. This solution contains approximately 30 millimoles per liter (mM) nitrogen. A 3/4 dilution, a 1/2 dilution, and a 1/4 dilution are prepared with 1% H_2SO_4 to provide a series of 4 standard solutions containing 30 mM nitrogen, 22.5 mM nitrogen, 15 mM nitrogen, and 7.5 mM nitrogen. Volumes of 0.020 ml of each of the 4 solutions, and of a 1% H_2SO_4 blank, are analyzed in duplicate as above, and their optical densities are plotted against nitrogen concentration to form a standard curve.

The optical densities of the unknown digest samples, the blank digest samples, and the control digest samples are converted to nitrogen concentrations in millimoles per liter (mM) by means of the standard curve. Because the same volume of 0.020 ml of sample solution is analyzed in every case, the nitrogen concentrations obtained are those of the samples directly.

The control digest provides a means for estimating and correcting for nitrogen recovery in the analytical procedure. A volume of 5.0 ml of the original control solution, described in the Kjeldahl digestion section, is digested to yield a final volume of 550 ml. Hence, it is diluted 110-fold in the digestion process. The theoretical concentration of nitrogen in the control digest is obtained by dividing the known nitrogen concentration of the original control solution of approximately 2.0 M by 110, to give a theoretical value of approximately 18 mM nitrogen. The actual nitrogen concentration of the control digest, measured colorimetrically, is then divided by the theoretical nitrogen concentration of the control digest to

yield a recovery fraction, which is usually somewhat less than 1.0. The measured nitrogen concentrations of the unknown sample digests are corrected for recovery by dividing them by the recovery fraction.

The corrected nitrogen concentrations of the unknown sample digests represent approximately 1 gram of net-body powder dissolved in 0.550 liters of final digest. Hence, the concentration of nitrogen in 1.000 gram of powder is given by the relationship:

$$\text{mM nitrogen/g powder} = \frac{0.55 \times \text{mM nitrogen in digest}}{\text{actual grams powder digested}}$$

The net-body nitrogen content in millimoles is obtained by multiplying the dry, fat-free net-body mass by the mM nitrogen/g powder. The net-body nitrogen content in grams is obtained by multiplying the millimoles by 0.01401 (atomic weight of nitrogen = 14.01). A reasonable approximation of the net-body protein content may be made by multiplying the net-body nitrogen content by the factor, 6.25.

Phosphorus Determination

Phosphorus determination in the Kjeldahl digests is based on the inorganic phosphate procedure of Grunbaum and Pace (5). The method depends on a timed color reaction produced by the serial addition of three reagent solutions to the sample solution to be analyzed. One of the reagent solutions must be freshly prepared for each day's analyses.

Ammonium heptamolybdate reagent is made by dissolving 37.5 grams of ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$) in 4.5 N sulfuric acid (H_2SO_4), to make 500 ml in a volumetric flask. The solution is allowed to stand overnight, and then is filtered. The second reagent is 10 N sulfuric acid (H_2SO_4). The third reagent, ferrous sulfate reagent must be prepared fresh on the day the

analyses are to be performed by dissolving 10.0 grams of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) and 1.0 ml of 4.5 N H_2SO_4 in distilled water, to make 100 ml in a volumetric flask.

A volume of 0.500 ml of the unknown diluted Kjeldahl digest is pipetted into a 17x100 mm capped polypropylene test tube (Falcon No. 2059). In rapid succession, 1.0 ml each of the ammonium heptamolybdate reagent, 10 N H_2SO_4, and ferrous sulfate reagent is added in that order, followed by the addition of 9.0 ml of distilled water. The tube is capped and the mixture is promptly shaken. The blue color is allowed to develop at room temperature for 15 min, a portion of the solution is transferred to a 1 cm cuvette, and the optical density at 660 nm is read in a spectrophotometer (Bausch and Lomb Spectronic 70).

Duplicate 0.500 ml samples of the blank and the control diluted Kjeldahl digests are analyzed in the same manner as above.

A standard curve for optical density as a function of phosphorus concentration is prepared by analyzing 4 solutions of monobasic potassium phosphate (KH_2PO_4) of different, known concentrations. These are prepared by dissolving an accurately weighed quantity of 0.55 grams of KH_2PO_4 in distilled water and making the final volume to 1.000 liter in a volumetric flask. This solution contains approximately 4 millimoles per liter (mM) phosphorus. A 3/4 dilution, a 1/2 dilution, and a 1/4 dilution are prepared with distilled water to provide a series of 4 standard solutions with concentrations of 4.0 mM phosphorus, 3.0 mM phosphorus, 2.0 mM phosphorus, and 1.0 mM phosphorus. Volumes of 0.500 ml of each of the 4 solutions, and of a distilled water blank, are analyzed in duplicate as above. The optical densities are plotted against the corresponding phosphorus concentration to form a standard curve.

The optical densities of the unknown digest samples, the blank digest samples, and the control digest samples are converted to phosphorus concentrations in millimoles per liter (mM) by means of the standard curve. Because

the same volume of 0.500 ml of sample solution is analyzed in every case, the phosphorus concentrations obtained are those of the samples directly.

The control digest provides a means for estimating and correcting for phosphorus recovery in the analytical procedure. A volume of 5.0 ml of the original control solution, described in the Kjeldahl digestion section, is digested to yield a final volume of 550 ml. Hence, it is diluted 110-fold in the digestion process. The theoretical concentration of phosphorus in the control digest is obtained by dividing the known phosphorus concentration of the original control solution of approximately 0.22 M by 110, to give a theoretical value of approximately 2.0 mM phosphorus. The actual phosphorus concentration of the control digest, measured colorimetrically, is then divided by the theoretical phosphorus concentration of the control digest to yield a recovery fraction, which is usually somewhat less than 1.0. The measured phosphorus concentrations of the unknown sample digests are corrected for recovery by dividing them by the recovery fraction.

The corrected phosphorus concentrations of the unknown sample digests represent approximately 1 gram of net-body powder dissolved in 0.550 liters of final digest. Hence, the concentration of phosphorus in 1.000 gram of powder is given by the relationship:

$$\text{mM phosphorus/g powder} = \frac{0.55 \times \text{mM phosphorus in digest}}{\text{actual grams powder digested}}$$

The net-body phosphorus content in millimoles is obtained by multiplying the dry, fat-free net-body mass by the mM phosphorus/g powder. The net-body phosphorus content in grams is obtained by multiplying the millimoles by 0.03097 (atomic weight of phosphorus = 30.97).

Potassium, Sodium, Calcium and Magnesium Determinations

The determination of K, Na, Ca and Mg concentrations in the unknown diluted Kjeldahl digest samples is based on the atomic-absorption spectrophotometry method of Sanui and Pace (11, 12). The method was developed expressly for cation measurements in Kjeldahl digests of biological materials. It incorporates the use of lanthanum to minimize the interference of sulfate and phosphate with atomic absorption calcium measurements, and the use of cesium to minimize the mutual interference of sodium and potassium. All glassware used in the atomic absorption analyses is rinsed with 1 M nitric acid, followed by thorough rinsing with distilled water and air-drying. All reagent solutions are stored in polyethylene bottles which have been cleaned in the same manner. Calibrated volumetric pipets are used throughout.

Lanthanum chloride reagent is made by adding 162.93 g lanthanum oxide (La_2O_3) to approximately 100 ml of distilled water in a 1-liter beaker. A volume of 320 ml of concentrated hydrochloric acid is added slowly with constant stirring to dissolve the La_2O_3. After cooling, the contents of the beaker are transferred quantitatively to a 1.000 liter volumetric flask and made up to volume with distilled water to yield 1.00 M $LaCl_3$ solution.

Cesium chloride reagent is made by dissolving 26.94 g cesium chloride (CsCl) in distilled water to make 1.000 liter in a volumetric flask. The resulting solution is 160 mM CsCl solution.

Sulfuric acid, 1.6 M, is made by slowly adding 88.8 ml of concentrated H_2SO_4, reagent grade, to approximately 500 ml of distilled water. After cooling, the contents of the beaker are transferred quantitatively to a 1.000 liter volumetric flask and made up to volume with distilled water.

Lanthanum-cesium reagent is prepared by adding 100 ml each of lanthanum chloride reagent, cesium chloride reagent, and 1.6 M sulfuric acid to a 1.000 liter volumetric flask and making up to volume with distilled water. The solution contains 100 mM lanthanum, 16 mM cesium, and 160 mM sulfuric acid.

A volume of 4.00 ml of the unknown diluted Kjeldahl digest sample is pipetted into a 17x100 mm capped polystyrene test tube (Falcon No. 2051). A volume of 2.50 ml of lanthanum-cesium reagent is added, followed by 3.50 ml of distilled water to make a total volume of 10.00 ml. The tube is capped and shaken. The solution contains 25 mM lanthanum and 4 mM cesium, and is ready for introduction into the atomic absorption spectrophotometer.

Volumes of 4.00 ml of the blank digest and of the control digest are prepared in duplicate in the same manner as the unknown digests for atomic absorption spectrophotometry.

A Perkin-Elmer Model 290B atomic absorption spectrophotometer equipped with Perkin-Elmer "Intensitron" lamps is used for the cation analyses. Perkin-Elmer lamp no. 303-6052 is used for potassium, which is measured at 766 nm with a slit width of 2 nm. Lamp no. 303-6065 is used for sodium, which is measured at 589 nm with a slit width of 0.7 nm. Lamp no. 303-6092 is used for both calcium and magnesium. Calcium is measured at 423 nm and magnesium is measured at 285 nm, both with a slit width of 0.7 nm. An acetylene-air flame burner (Perkin-Elmer no. 290-1169), with a 0.5x52 mm aperture and supplied by 8 pounds per square inch acetylene pressure and 40 pounds per square inch air pressure, is used parallel to the light beam. The sample uptake rate is set at 3.2 ml/min to provide maximal signal intensity.

A zero-setting solution is made up by adding 25 ml of lanthanum chloride reagent, 25 ml of cesium chloride reagent, and 25 ml of 1.6 M sulfuric acid to a 1-liter volumetric flask, and making up to volume with distilled water. The absorbance reading of the spectrophotometer is set to zero while this

solution is being aspirated into the flame.

A set of standard solutions of 6 different concentrations of the cation to be measured, encompassing the expected concentration range, is prepared so that each also contains concentrations of 25 mM lanthanum, 4 mM cesium and 40 mM sulfuric acid. The absorbance reading of the spectrophotometer is set to 100 while the highest-concentration standard solution is being aspirated into the flame. The absorbance of the other 5 standard solutions is then measured to insure linearity of response of the spectrophotometer, and to provide closely-bracketing standard values for the unknown sample to be measured.

The unknown digest sample is aspirated into the flame, and the absorbance value is recorded. The standard solution with the next-lower absorbance is aspirated into the flame, and the absorbance value is recorded. The standard solution with the next-higher absorbance is then aspirated into the flame, and the absorbance value is recorded. This bracketing procedure is used with each unknown sample measured, to compensate for drift in the spectrophotometer. The cation concentration in the unknown samples may be computed from the relationship:

$$\text{Sample Conc} = \frac{(\text{HS Conc} - \text{LS Conc})}{(\text{HS Abs} - \text{LS Abs})} \times (\text{Sample Abs} - \text{LS Abs}) + \text{LS Conc}$$

where Sample Conc is the cation concentration (mM) in the unknown sample, HS Conc is the cation concentration (mM) in the next-higher standard solution, LS Conc is the cation concentration (mM) in the next-lower standard solution, Sample Abs is the absorbance reading for the unknown sample, and HS Abs and LS Abs are the absorbance readings for the two bracketing standard solutions.

The set of standard solutions for <u>potassium</u> measurements is made by dissolving approximately 7.456 grams, accurately weighed, of reagent-grade

potassium chloride (KCl) in distilled water to make 1.000 liter in a volumetric flask. The resulting potassium standard stock solution is 100 mM KCl. Into each of six 1-liter volumetric flasks the following volumes of 100 mM KCl stock solution are pipetted: 0.50 ml, 1.00 ml, 1.50 ml, 2.00 ml, 3.00 ml, and 4.00 ml. A volume of 25.0 ml of each of 1 M lanthanum chloride reagent, 160 mM cesium chloride reagent, and 1.6 M sulfuric acid is added to each flask. Distilled water is added to make each flask up to 1.000 liter final volume. The resulting 6 potassium standard solutions range in concentration from 0.050 mM to 0.400 mM.

The set of standard solutions for sodium measurements is made by dissolving approximately 5.844 grams, accurately weighed, of reagent-grade sodium chloride (NaCl) in distilled water to make 1.000 liter in a volumetric flask. The resulting sodium standard stock solution is 100 mM NaCl. A volume of 10.0 ml of 100 mM NaCl stock solution is diluted to 100.0 ml with distilled water in a volumetric flask to make 10 mM NaCl. Into each of six 1-liter volumetric flasks the following volumes of 10 mM NaCl solution are pipetted: 1.00 ml, 2.50 ml, 5.00 ml, 10.00 ml, 15.00 ml, and 20.00 ml. A volume of 25 ml each of 1 M lanthanum chloride reagent, 160 mM cesium chloride reagent, and 1.6 M sulfuric acid is added to each flask. Distilled water is added to make each flask up to 1.000 liter final volume. The resulting 6 sodium standard solutions range in concentration from 0.010 mM to 0.200 mM.

A set of standard solutions for calcium measurements is made by dissolving approximately 10.009 grams, accurately weighed, of reagent-grade calcium carbonate ($CaCO_3$) in a minimal volume of concentrated hydrochloric acid (HCl) and making up to 1.000 liter in a volumetric flask with distilled water. The resulting calcium standard stock solution is 100 mM $CaCl_2$. Into each of six 1-liter volumetric flasks the following volumes of 100 mM $CaCl_2$ solution are

pipetted: 1.00 ml, 2.00 ml, 3.00 ml, 5.00 ml, 10.00 ml and 20.00 ml. A volume of 25 ml each of 1 M lanthanum chloride reagent, 160 mM cesium chloride reagent, and 1.6 M sulfuric acid is added to each flask. Distilled water is added to make each flask up to 1.000 liter final volume. The resulting 6 calcium standard solutions range in concentration from 0.10 mM to 2.00 mM.

The set of standard solutions of magnesium measurements is made by dissolving approximately 2.431 grams, accurately weighed, of reagent-grade magnesium metal shavings in a minimal volume of concentrated hydrochloric acid (HCl) and making up to 1.000 liter in a volumetric flask with distilled water. The resulting magnesium stock solution is 100 mM $MgCl_2$. A volume of 10.0 ml of 100 mM $MgCl_2$ stock solution is diluted to 100.0 ml with distilled water in a volumetric flask to make 10 mM $MgCl_2$. Into each of six 1-liter volumetric flasks the following volumes of 10 mM $MgCl_2$ stock solution are pipetted: 1.00 ml, 2.00 ml, 4.00 ml, 6.00 ml, 8.00 ml, and 10.00 ml. A volume of 25 ml each of 1 M lanthanum chloride reagent, 160 mM cesium chloride reagent, and 1.6 M sulfuric acid is added to each flask. Distilled water is added to make each flask up to 1.000 liter final volume. The resulting 6 magnesium standard solutions range in concentration from 0.010 mM to 0.100 mM.

The cation concentrations of the blank digest samples and the control digest samples are measured in the same way as for the unknown digest samples. The control digest provides a means for estimating and correcting for cation recoveries in the analytical procedure. A volume of 5.0 ml of the original control solution, described in the Kjeldahl digestion section, is digested to yield a final volume of 550 ml. Hence, it is diluted 110-fold in the digestion process. A volume of 4.00 ml of the control digest is further diluted 2.5-fold to 10.00 ml when the sample is prepared for atomic absorption

spectrophotometry. Hence, the theoretical cation concentrations of the control digest samples analyzed may be computed from the known cation concentrations of the original control solution by dividing them by 110x2.5, or 275. The actual concentration of each cation of the control digest samples, measured by atomic absorption, is then divided by the theoretical concentration of that cation to yield a recovery fraction. The measured cation concentrations of the unknown sample digests are corrected for recovery by dividing each by the appropriate recovery fraction. The recovery fractions for potassium, calcium and magnesium are usually somewhat less than 1.0; whereas, the recovery fractions for sodium are usually somewhat greater than 1.0. Presumably, the latter represents the effect of leaching of sodium from the glass Kjeldahl flasks during the digestion process.

The corrected cation concentrations of the unknown sample digests represent approximately 1 gram of net-body powder dissolved in 0.550 liters of final digest, which is then diluted 2.5-fold for atomic absorption analysis. Hence, the concentration of each cation in 1.000 gram of powder is given by the relationship:

$$\text{mM cation/g powder} = \frac{0.55 \times 2.5 \times \text{mM cation by atomic absorption}}{\text{actual grams powder digested}}$$

The net-body content of each cation is obtained by multiplying the dry, fat-free, net-body mass by the mM cation/g powder in each case.

The net-body potassium content in grams is obtained by multiplying the net-body potassium millimoles by 0.03910 (atomic weight of potassium = 39.10). The net-body sodium content in grams is obtained by multiplying the net-body sodium millimoles by 0.02299 (atomic weight of sodium = 22.99). The net-body calcium content in grams is obtained by multiplying the net-body calcium millimoles by 0.04008 (atomic weight of calcium = 40.08). The

net-body magnesium content in grams is obtained by multiplying the net-body magnesium millimoles by 0.02431 (atomic weight of magnesium = 24.31).

Creatine Determination

The determination of net-body creatine content is based on a hot-acid extraction procedure described originally by Folin (2), and on the measurement of creatinine concentration in the acid extract by the method of Grunbaum and Pace (5). The creatine is converted to creatinine by the hot acid during the extraction process.

Somewhat more than 1 gram of dried, defatted, net-body powder is placed in a desiccator overnight. Approximately 1.0 gram of the desiccated powder is weighed out accurately to 1 mg in a disposable plastic weighing tray (Scientific Products Catalog No. B2045-10), using a Mettler Model B6, semi-micro analytical balance. The powder is transferred quantitatively to a 25x200 mm Pyrex screw-cap culture tube (Corning No. 9826), and 20.0 ml of 1 N sulfuric acid (H_2SO_4) are pipetted into the tube. The tube is tightly capped, and is gently swirled to wet the powder. The tube is placed in a boiling-water bath for 1 hr, and is then cooled under tap water before opening. The contents are poured into a 30 ml screw-capped polycarbonate centrifuge tube (Scientific Products Catalog No. C4049-30), and the tube is centrifuged at 1,000 *g* for 15 min. A volume of 5 ml of the clear supernatant acid extract solution is carefully removed by pipet, and is transferred to a 17x100 mm capped polystyrene test tube (Falcon No. 2051) for subsequent analysis.

Duplicate blank sample extracts are prepared in parallel with the unknown animal powder samples. A volume of 20.0 ml of 1 N sulfuric acid is pipetted into each of two 25 x 200 mm Pyrex screw-cap culture tubes, which

are then treated in the same manner as the unknown samples to yield blank sample extracts.

Duplicate control solution extracts are also prepared in parallel with the unknown animal powder samples. A control solution is prepared by dissolving approximately 0.168 grams, accurately weighed, of reagent-grade creatine monohydrate ($H_2NC(:NH)N(CH_3)CH_2CO_2H \cdot H_2O$), molecular weight 149.15, in 1 N sulfuric acid to make 250.0 ml final volume in a volumetric flask. The control solution contains approximately 4.5 mM creatine. A volume of 20.0 ml of the control solution is pipetted into each of two 25x200 mm Pyrex screw-cap culture tubes, which are also treated in the same manner as the unknown samples to yield control sample extracts.

A 1% solution of picric acid is made by dissolving 1 gram of reagent-grade picric acid ($(O_2N)_3C_6H_2OH$) in distilled water to make 100 ml final volume. A 10% solution of sodium hydroxide is made by dissolving 10 grams of reagent-grade sodium hydroxide (NaOH) in distilled water to make 100 ml. Both solutions should be freshly made on the day of use.

A volume of 0.050 ml of the extract solution to be analyzed for creatinine concentration is pipetted into a 17x100 mm capped polystyrene test tube (Falcon No. 2051), followed by the addition of 2.0 ml of the 1% picric acid and 0.150 ml of the 10% sodium hydroxide. The tube is capped, shaken, and allowed to stand for 30 min. A volume of 7.8 ml of distilled water is added to the tube, which is recapped and inverted 3 times for thorough mixing of the contents. A portion of the orange-colored solution is transferred to a 1 cm cuvette, and the optical density at 490 nm is read in a spectrophotometer (Bausch and Lomb Spectronic 70).

A standard curve for optical density as a function of creatinine concentration is prepared by analyzing 4 solutions of creatinine of

different, known concentrations. These are prepared by dissolving an accurately weighed quantity of approximately 0.453 grams of reagent-grade creatinine, molecular weight 113.12, in 1 N sulfuric acid to make 500 ml final volume in a volumetric flask. This solution contains approximately 8.0 millimoles per liter (mM) creatinine. A 3/4 dilution, a 1/2 dilution, and a 1/4 dilution are prepared with 1 N sulfuric acid to provide a series of 4 standard solutions with creatinine concentrations of 8.0 mM, 6.0 mM, 4.0 mM, and 2.0 mM. Volumes of 0.050 ml of each of the 4 solutions, and of a 1 N sulfuric acid blank, are analyzed in duplicate as described in the preceding paragraph. The optical densities are plotted against the corresponding creatinine concentration to form a standard curve.

The optical densities of the unknown sample extracts, the blank sample extracts, and the control sample extracts are converted to creatinine concentrations in millimoles per liter (mM) by means of the standard curve. Because the same volume of 0.050 ml of sample solution is analyzed in every case, the creatinine concentrations obtained are those of the samples directly.

The control sample extract provides a means for estimating and correcting for creatinine recovery in the analytical procedure. Since the conversion of creatine to creatinine in hot acid is mole-for-mole, the measured concentration of creatinine in the control sample extract is divided by the known concentration of creatine in the original control solution to yield a recovery fraction. The measured creatinine concentrations of the unknown sample extracts are corrected for recovery by dividing them by the recovery fraction.

The corrected creatinine concentration of the unknown sample extracts represents approximately 1 gram of net-body powder extracted by 0.020 liters of 1 N sulfuric acid. Hence, the concentration of creatine originally

present in 1.000 gram of powder is given by the relationship:

$$\text{mM creatine/g powder} = \frac{0.020 \times \text{mM creatinine in extract}}{\text{actual grams powder extracted}}$$

The net-body creatine content in millimoles is obtained by multiplying the dry, fat-free, net-body mass by the mM creatine/g powder. The net-body creatine content in grams is obtained by multiplying the millimoles by 0.13114 (molecular weight of creatine = 131.14).

Hunter (6) has pointed out that about 98% of all the creatine in the body is found in the skeletal musculature of the body, and that in mammals the creatine concentration is about 0.004 g/g muscle. However, there appears to be variation both with age of the animal and with species. Therefore, a careful appraisal of the creatine concentration of muscle as a function of age and species will be necessary before body creatine content may be used as a reliable measure of the body muscle mass. Such a study is presently in progress in our laboratory.

COMPUTATION OF BODY COMPOSITION PARAMETERS FROM ANALYTICAL RESULTS

The results of the elemental analyses of the Kjeldahl digest of the dry, defatted net-body powder from the example 82-day-old male rat are given in Table 2. The second column of the table shows the concentration of each element in millimoles per gram of powder, as determined by the procedures described in the preceding section. Each of these values is multiplied by the dry, fat-free, net-body mass of the animal from Table 1, 79.52 g, to yield the millimoles of each element in the net-body mass, and shown in column 3. The values in column 3 are multiplied by the appropriate milliatomic weight to provide the mass in grams of each element contained in the fat-free net body mass, as listed in column 4. Finally, the percentage of the fat-free net body mass represented by each element is given in column 5.

Table 2. Elemental analyses of dry, defatted, net-body powder, and elemental body content values for example animal of 308.96 g fat-free net body mass and 79.52 g dry, fat-free, net body mass.

Element	Concentration in Body Powder (mM/g)	Net-Body Elemental Content (mM)	Net-Body Elemental Content (g)	Percentage of Fat-Free Net Body Mass (%)
Nitrogen	8.951	711.8	9.97	3.23
Calcium	0.9362	74.45	2.98	0.965
Phosphorus	0.7080	56.30	1.744	0.564
Potassium	0.3023	24.04	0.940	0.304
Sodium	0.2011	15.99	0.368	0.119
Magnesium	0.0571	4.54	0.1104	0.0357

Moore et al. (7) have shown that body potassium content yields a good estimate of the body cell mass. Based on the fact that at least 95% of the body potassium is contained intracellularly, on the premise that intracellular potassium concentration is maintained at an essentially constant value of 146 mM over a wide range of physiological states, and on the assumption that the average body tissue cell contains 73% water, the body cell mass may be computed as

$$\textit{Body Cell Mass, g} = 228(\textit{Body Potassium Mass, g}).$$

For the example animal value in Table 2

$$\text{Body Cell Mass} = 228 \times 0.940 = 214.32 \text{ g}.$$

On the assumption that intracellular water constitutes 73% of the body cell mass, it may be computed as

$$\textit{Intracellular Water Mass, g} = 0.73(\textit{Body Cell Mass, g})$$

For the example animal it is

$$\text{Intracellular Water Mass} = 0.73 \times 214.32 = 156.45 \text{ g}$$

Total body water represents the sum of the intracellular water and extracellular water, so that the latter may be obtained as

$$\textit{Extracellular Water Mass, g} = (\textit{Body Water Mass, g}) - (\textit{Intracellular Water Mass, g})$$

The body water mass for the example animal was given in Table 1 as 229.44 g, so that

$$\text{Extracellular Water Mass} = 229.44 - 156.45 = 72.99 \text{ g}.$$

The average composition of the body bone mineral has been postulated by Neuman and Neuman (8) to be best represented by the empirical formula

$$[Ca_9^{++}(H_3O^{+})_2(PO_4^{\equiv})_6(OH^{-})_2][Ca^{++}\cdot Mg_{0.3}^{++}\cdot Na_{0.3}^{+}\cdot CO_3^{=} \cdot \text{citrate}_{0.3}^{\equiv}].$$

The bone mineral complex has an equivalent molecular weight of 1,173.63 g, of which 400.80 g are calcium. Inasmuch as the skeleton accounts for at

least 98% of the body calcium (14), for all practical purposes the body bone mineral mass may be computed as

$$\text{Body Bone Mineral Mass, g} = \frac{1{,}173.63}{400.80} \text{ (Body Calcium Mass, g)}$$

or

$$\textit{Body Bone Mineral Mass, g} = 2.93 \textit{ (Body Calcium Mass, g).}$$

For the example animal value in Table 2

$$\text{Body Bone Mineral Mass} = 2.93 \times 2.98 = 8.73 \text{ g.}$$

The total body mass may be regarded as comprising the body cell mass, which accounts for the body oxygen consumption and metabolic energy release, and the inert total body mass (9), which includes hair, gut contents, body fat, extracellular fluid, bone mineral, and the intercellular organic matrix of fibers and amorphous ground substance of connective tissues. It may be computed as

$$\textit{Inert Total Body Mass, g} = \textit{(Total Body Mass, g)} - \textit{(Body Cell Mass, g)}$$

For the example animal it is

$$\text{Inert Total Body Mass} = 372.43 - 214.32 = 158.11 \text{ g}$$

All the constituents of the inert total body mass, save for the intercellular organic matrix of the connective tissues, are evaluated by the procedures described. The hair and gut content masses for the example animal are given in Fig. 3 as 6.50 g and 12.62 g, respectively, the body fat mass is given in Fig. 4 as 44.35 g, the extracellular water mass was computed above as 72.99 g, and the bone mineral mass was computed to be 8.73 g. Hence, the intercellular matrix mass may be calculated as the difference between the sum of these masses and the inert body mass, or 12.92 g.

The inert net body mass may be computed as

$$\textit{Inert Net Body Mass, g} = \textit{(Net Body Mass, g)} - \textit{(Body Cell Mass, g)}$$

For the example animal it is

Inert Net Body Mass = 353.31 - 214.32 = 138.99 g,

and represents the sum of the body fat mass, extracellular water mass, bone mineral mass, and intercellular matrix mass.

The inert fat-free net body mass is obtained as

Inert Fat-Free Net Body Mass, g = (Fat-Free Net Body Mass, g) - (Body Cell Mass, g)

For the example animal it is

Inert Fat-Free Net Body Mass = 308.96 - 214.32 = 94.64 g,

and represents the sum of the extracellular water mass, bone mineral mass, and intercellular matrix mass.

For some purposes it is useful to examine values for the dry, inert body mass. However, it is necessary to distinguish between the dry, inert total body mass, which includes the hair and dry gut contents, and the dry, inert net body mass, or the dry, inert fat-free net body mass.

The dry total body mass is computed as

Dry Total Body Mass, g = (Total Body Mass, g) - (Net Body Water, g) - (Gut Contents Water, g)

For the example animal, it is

Dry Total Body Mass = 372.43 - 229.44 - 9.88 = 133.11 g.

The dry, inert total body mass is then obtained as

Dry Inert Total Body Mass, g = (Dry Total Body Mass, g) - (Dry Body Cell Mass, g).

For the example animal, it is

Dry, Inert Total Body Mass = 133.11 - 57.87 = 75.24 g.

The dry, inert, total body mass also represents the sum of the masses for hair, dry gut contents, net body fat, bone mineral, and intercellular matrix.

The *dry, inert net body mass* comprises the sum of the net body fat mass, the bone mineral mass, and the intercellular matrix mass. Alternatively it may be obtained as the difference between the inert net body mass and the extracellular water mass. For the example animal the dry, inert net body mass is 66.00 g.

Perhaps the most useful of these dry, inert body mass parameters is the *dry, inert fat-free net body mass*, which is simply the sum of the bone mineral mass and the intercellular matrix mass. For the example animal it is 21.65 g.

Table 3 summarizes all the body composition parameters derived by application of the procedures described in the foregoing sections to the 82-day-old male rat used as the example animal. The results provide a quantitative biochemical dissection of a mammal based on both functional and morphological considerations, and permit further permutations of body composition parameters to be examined as well.

Table 3. Summary of body composition parameters derived by application of the analytical procedures to the example 82-day-old male rat.

	Mass (g)	Total Body Mass (%)	Net Body Mass (%)	Fat-Free Net Body Mass (%)
Total Body	372.43	100.00	--	--
Dry Total Body	133.11	35.74	--	--
Hair	6.50	1.74	--	--
Gut Contents	12.62	3.39	--	--
Dry Gut Contents	2.74	0.74	--	--
Gut Contents Water	9.88	2.65	--	--
Net Body	353.31	94.87	100.00	--
Dry Net Body	123.87	33.26	35.06	--
Net Body Water	229.44	61.61	64.94	74.26
Net Body Fat	44.35	11.91	12.55	--
Fat-Free Net Body	308.96	82.96	87.45	100.00
Dry Fat-Free Net Body	79.52	21.35	22.51	25.74
Body Cells	214.32	57.55	60.66	69.37
Dry Body Cells	57.87	15.54	16.38	18.73
Intracellular Water	156.45	42.01	44.28	50.64
Extracellular Water	72.99	19.60	20.66	23.62
Bone Mineral	8.73	2.34	2.47	2.83
Intercellular Matrix	12.92	3.47	3.66	4.18
Inert Total Body	158.11	42.45	--	--
Dry Inert Total Body	75.24	20.20	--	--
Inert Net Body	138.99	37.32	39.34	--
Dry Inert Net Body	66.00	17.72	18.68	--
Inert Fat-Free Net Body	94.64	25.41	26.79	30.63
Dry Inert Fat-Free Net Body	21.65	5.81	6.13	7.01
Net Body Nitrogen	9.97	2.68	2.82	3.23
Net Body Protein	62.31	16.73	17.64	20.17
Net Body Calcium	2.98	0.80	0.84	0.96
Net Body Phosphorus	1.74	0.47	0.49	0.56
Net Body Potassium	0.94	0.25	0.27	0.30
Net Body Sodium	0.37	0.10	0.10	0.12
Net Body Magnesium	0.11	0.03	0.03	0.04

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